PCT WORLD INTELLECTUAL I
Internatio

INTERNATIONAL APPLICATION PUBLISHED UN WO 9605809A1

(51) International Patent Classification 6 :		(11) International Publication Number: WO 96/05809
A61K 9/14, 9/19, 47/26, 47/22	A1	(43) International Publication Date: 29 February 1996 (29.02.96)

(21) International Application Number: PCT/GB95/01967

(22) International Filing Date: 18 August 1995 (18.08.95)

(30) Priority Data:
08/293,157 19 August 1994 (19.08.94) US

(71) Applicant *(for all designated States except US)*: QUADRANT HOLDINGS CAMBRIDGE LIMITED [GB/GB]; Maris Lane, Trumpington, Cambridge CB2 2SY (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: COLACO, Camilo [GB/GB]; 107 Foster Way, Trumpington, Cambridge CB2 2JN (GB). ROSER, Bruce, Joseph [GB/GB]; 4 Archway Court, Barton Road, Cambridge CB3 9LW (GB). SEN, Shevanti [IN/GB]; 4 Archway Court, Barton Road, Cambridge CB3 9LW (GB).

(74) Agent: ABLEWHITE, Alan, James; Marks & Clerk, 57-60 Lincoln's Inn Fields, London WC2A 3LS (GB).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IMPROVED METHOD FOR STABILIZATION OF BIOLOGICAL SUBSTANCES DURING DRYING AND SUBSEQUENT STORAGE AND COMPOSITIONS THEREOF

(57) Abstract

A method of increasing stability of biologial substances during drying comprises adding to an aqueous solution or suspension of the substances and a carbohydrate excipient in an amount sufficient to stabilize the substances, inhibitors of the Maillard reaction in an amount effective to substantially prevent condensation of amino groups and reactive carbonyl groups and drying the resulting composition.

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SI	Slovenia
SK	Slovakia
SN	Senegal
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

IMPROVED METHOD FOR STABILIZATION OF BIOLOGICAL SUBSTANCES DURING DRYING AND SUBSEQUENT STORAGE AND COMPOSITIONS THEREOF

Field of the Invention

The present invention relates generally to methods for increasing stabilization of biological substances during drying and storage of dry formulations. Compositions of stabilized biological substances are also provided.

Background of the Invention

Storage stability is the ultimate goal for dried formulations of biological substances. Dried formulations are preferred to aqueous formulations as the water, a nucleophile in hydrolysis reactions and a plasticizer, increases the molecular mobility of reactive chemical species, making aqueous formulations of biological substances inherently less stable than their dry counterparts. This increased stability of dry formulations has focused attention on techniques of drying and led to the development of freeze-drying as a popular method of water removal. Pikal (1990a) Biopharm. 3:18-27; Pikal (1990b) Biopharm. 3:26-30; and Franks (1990) Cryoletters 11:93-100. However, despite its widespread use, many freeze-dried products are still unstable at ambient temperatures. Carpenter and Crowe (1988) Cryobiol. 25:244-255; and Crowe et al. (1990) Cryobiol. 27:219-231. Detailed theoretical analyses of the physicochemical events during freeze-drying have led to a substantial literature on the use of lyoprotectants as stabilizing excipients. Pikal

(1990b); Carpenter and Crowe (1988); Crowe et al. (1990); and Levine and Slade (1988) Pure Appl. Chem. 60:1841-1864. Various carbohydrates have been advocated as stabilizing excipients in freeze-drying, and these are proposed to act via the generation of an amorphous, glassy, solid state in the freezing step. Pikal (1990b); Franks (1990); Levine and Slade (1988); and Franks et al. (1992) U.S. Patent No. 5,098,893. Nevertheless, the freezing step remains a major variable, as evidenced by the equivocal values for the experimentally measured glass transition temperature of the maximally freeze-concentrated unfrozen matrix (T'g) for various carbohydrate excipients. Franks (1990); Levine and Slade (1988); Ablett et al. (1992) J. Chem. Soc. Faraday Trans. 88:789-794; and Roos (1993) Carbo. Res. 238:39-48.

Exposure to temperatures above the glass transition or collapse during freeze drying can result in significant activity losses. Furthermore, freezing is thought to be the major cause of protein damage during freeze-drying. Due to the loss of activity on freeze drying, recent attention has focused on the techniques of ambient temperature drying. These not only eliminate the freezing step but are more rapid and energy-efficient in the removal of water during drying. Crowe et al. (1990); Roser (1991) Biopharm. 4:47-53; Roser and Colaco (1993) New Scientist 138:24-28; Blakely et al. (1990) The Lancet 336:854-55; Colaco et al. (1990) Biotech. Intl. pp. 345-350, Century Press, London; Colaco et al. (1992) Biotech. 10:1007-1011; Franks (1991) Biopharm. 14:38-55; Franks and Hatley (1993) in "Stability and stabilization of enzymes", eds. van den Tweel et al., Elsevier, Amsterdam (1993) pp. 45-54; and Franks (1994) Bio/Tech. 12:253-256; Roser, U.S. Patent No. 4,891,319; and Roser et al. (1991) WO91/18091.

All references cited herein, both supra and infra, are hereby incorporated herein by reference.

Stabilization of dried biological substances, particularly at ambient or higher temperatures, remains a challenge. One carbohydrate, trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), has been found to be uniquely potent in prolonging shelf life of dried proteins and other biological materials for prolonged periods at ambient or higher temperatures. Stability has been assessed by recovery of biological activity upon rehydration. Roser (1991); Roser and Colaco (1993); Blakely et al. (1990); Colaco et al. (1990); Colaco et al. (1992); and Carpenter and Crowe (1988) Cryobiol. 25:459-470. Studies of other sugars, polyhydric alcohols and oligosaccharides under conditions identical to those in which trehalose provides protein stability, showed that this degree of stabilization is unique to trehalose. Some of these excipients are partially protective, however, in that they protect the biomolecules from damage during the drying process itself and confer more limited tolerance to high temperatures. Crowe et al. (1990); Roser (1991); Colaco et al. (1990); Crowe et al. (1987); Carpenter et al. (1987); and Carpenter and Crowe (1988).

There are two main hypotheses that have been postulated with respect to the molecular mechanism by which trehalose stabilizes biological molecules. Clegg (1985) in Membranes, metabolism and dry organisms, ed. Leopold, Cornell Univ. Press, Ithaca, NY, pp. 169-187; Burke (1985) in Membranes, metabolism and dry organisms ed. Leopold, Cornell Univ. Press, Ithaca, NY, pp. 358-363; Green and Angell (1989) J. Phys. Chem. 93:2280-2882; Levine and Slade (1992) Biopharm. 5:36-40; and Crowe et al. (1990) Biopharm. 6:28-37, The water replacement theory states that, being a polyol, trehalose can make multiple external

hydrogen bonds which could replace the essential structural water molecules that are hydrogen-bonded to biomolecules and thus maintain their molecular structure. Clegg (1985); and Crowe et al. (1993). The glassy state theory postulates that, as the drying trehalose solutions undergo glass transformation, this results in an amorphous continuous phase in which molecular motion, and thus degradative molecular reactions, are kinetically insignificant. Burke (1985); Green and Angell (1989); and Levine and Slade (1992). Results previously obtained and those described herein are not consistent with either hypothesis being a sufficient sole explanation for the mechanism of action of trehalose.

The water replacement theory suggests that, as polyols, sugars other than trehalose should also be effective as stabilizing excipients, and, if the specific spatial combinations of hydroxyl groups are the crucial feature, then glucose should be as effective as trehalose. However, results obtained previously and presented herein show that glucose, a polyol, is among the least effective of a variety of sugars tested (see Table 1), and none of the other polyols tested was found to be as effective as trehalose (Fig. 1 and Table 1). Furthermore, if molecular mimicry of water was important, as might be expected for water replacement, then scylloinositol (with all its hydroxyl groups being axial) should, in theory, be the most effective carbohydrate, but it is among the least effective in practice. Thus, the water replacement theory cannot be a complete explanation for the mechanism of action of trehalose.

Similarly, the glassy state theory alone cannot explain the stability conferred by trehalose. In high temperature storage stability data reported in Fig. 1 above, the glass transition temperatures of the samples dried in trehalose to a water content of 2.6-

3.6% were all below 37°C as measured by differential scanning calorimetry. Thus, their stability persists at well above their glass transition temperatures, and although the glassy state may be important in other systems, contrary to accepted belief (Franks et al. (1992); and Franks (1994)), it appears not to be a factor in the long-term high temperature stability of biomolecules dried in trehalose.

Although current belief is that protein stability depends almost solely on glass transition temperature, several possible contributing reactions that decrease protein stability in the presence of sugars have been proposed. Franks (1994). These possible reactions have not been analyzed and are thought to contribute little, if any, to protein instability. Franks et al. (1994); and Akers and Nail (1994) Pharm. Tech. 18:26-36.

Summary of the Invention

The present invention encompasses methods of increasing stability of biological substances during drying and the dried compositions derived therefrom. One method includes drying the biological substances in the presence of a carbohydrate excipient in an amount effective to stabilize the dried biological substance and an inhibitor of the Maillard reaction in an amount effective to substantially prevent condensation of amino groups and reactive carbonyl groups. Another method includes increasing storage stability of dried biological substances by storing the substances in the presence of a carbohydrate excipient in an amount sufficient to stabilize the biological substance and an inhibitor of the Maillard reaction in an amount sufficient to substantially prevent condensation of amino groups and reactive carbonyl groups. Compositions of biological

substances, carbohydrates and inhibitors of the Maillard reaction are also included.

Brief Description of the Drawings

Fig. 1. Accelerated aging study on the restriction enzyme *Pst* I.

Fig. 2. Non-enzymatic browning of the samples studied for accelerated aging.

Fig. 3. Schematic of the Maillard reaction.

Fig. 4. Browning of alkaline phosphatase dried in the presence of fructose and glucose.

Fig. 5. HPLC analysis of glucagon dried in the presence of glucose or trehalose.

Fig. 6. Effect of residual water content on chemical reactions in model excipients.

Fig. 7. Activity of alkaline phosphatase upon drying and storage in glucose and increasing amounts of lysine.

Description of the Invention

Previous work of the stabilization of dry protein formulations in the presence of carbohydrate excipients has emphasized the ability of stabilizing excipients to replace structural water and/or provide an amorphous or glassy solid matrix in the dry state. These studies have failed to realize the effect and nature of chemical reactions during drying and storage subsequent to drying. As shown in the examples presented herein, these reactions, which may begin during drying, are greatly enhanced during storage. These examples show that the Maillard reaction is the main chemical reaction occurring during drying and upon storage of dried biological substances in the presence of carbohydrate excipients. The methods and formulations described herein prevent the Maillard reaction during storage and thus increase shelf life of stored, dried biological substances.

The Maillard reaction is actually a cascade of chemical reactions initiated by the reversible spontaneous condensation of reactive carbonyl and amino groups to form a Schiff's base. The activation energy of the initial condensation to form the Schiff's base is only on the order of 10-15 kcal, is reversible in the presence of water, and the equilibrium is largely in favor of the reactants in aqueous environments. Reynolds (1965) Adv. Food Res. 14:167-283; Finot et al. (1990) The Maillard Reaction in Food Processing, Human Nutrition and Physiology Birkhauser, Basel; and Lendl and Schleicher (1990) Ang. Chem. 29:565-594. A schematic of the components of the Maillard reaction is presented in Figure 3. Formation of the Schiff's base is a cascade of subsequent reaction resulting in the cross-linking of the proteins involved and the generation of brown melanoid pigments. The subsequent spontaneous Amadori or Heyns rearrangement of the Schiff's base is irreversible and triggers a complex series of reactions that ultimately result in the production of brown melanoidin pigments and both fragmentation and cross-linking of the proteins involved. In the food industry, the Maillard reaction has been widely studied, as it is one of several causes of spoilage, especially of dried food products, during storage. It has even been observed during refrigerated storage of foodstuffs with high protein and sugar contents. The Maillard reaction is a particular problem with dry foodstuffs, as the equilibrium of the reaction is forced towards the formation of the Schiff's base by the loss of water, and many of the subsequent reactions are accelerated at low water activities. Lendl and Schleicher (1990); and Nursten (1986) in Maillard browning reactions in dried foods in concentration and drying of foodstuffs, ed. McCarthy Elsevier Applied Science, London.

The present invention encompasses methods of increasing stability of biological substances during drying and storage and dried compositions thereof with increased storage stability. In the methods and compositions, although singular forms may be used, more than one carbohydrate, more than one biological substance and more than one inhibitor of the Maillard reaction may be present. Determination of the effective amounts of these compounds is within the skill of one in the art.

The biological substances may be derived from natural sources or chemically synthesized. Typically they are biologically active, or are able to exert a biological effect. However, intermediates or combinations of active compounds and intermediates are also encompassed. Likewise, formulations of carbohydrates and inhibitors of the Maillard reaction into which biological substances are to be incorporated are encompassed by the present invention. The biological substances may be any known in the art and include, but are not limited to, subcellular compositions, cells, viruses and molecules including, but not limited to, those containing free amino, imino and guanidino side chains, although singular forms may be used. Such molecules include, but are not limited to, pharmaceutical agents, lipids, organics, peptides, proteins, hormones (peptide, steroid and corticosteroid), oligosaccharides, synthetic peptides, D and L amino acid polymers, nucleic acids, protein nucleic acid hybrids and small molecules. Suitable proteins include, but are not limited to, enzymes, biopharmaceuticals, growth hormones, growth factors, monoclonal antibodies, and cytokines. Organics include, but are not limited to, pharmaceutically active chemicals with amino, imino or guanidino groups.

The method of drying may be any known in the art and includes, but is not limited to, freeze drying, spray drying, fluidized-bed drying, drum drying, drying at ambient temperature and atmospheric pressure, drying at ambient temperature and decreased pressure, drying at elevated temperatures and atmospheric pressure and drying at elevated temperatures and decreased pressure. In the case of freeze drying, the samples are typically frozen at -70 to -30°C and dried at a condenser temperature of -50 to -80°C, although any suitable temperature may be used. Freezing can be performed by any method known in the art including, but not limited to, immersing in liquid nitrogen, placing in a freezer which may be at -4°C to -80°C and a dry ice and alcohol freezing bath. In the case of ambient or elevated temperature drying, any temperature above freezing may be used, provided it is not so high that the biological substance becomes denatured; preferably the temperatures are less than 80°C, more preferably less than 65°C and most preferably 20-50°C. Preferably the temperature is above ambient to 50°C. In the case of spray drying, the temperature range is less than 250°C, more preferably in the range of 150-220°C, and most preferably, 180-200°C.

As used herein, with respect to storage or drying, ambient, or "room temperature" is generally about 20°C and elevated temperatures are all those above freezing. Temperatures "above ambient" are those greater than 20°C.

As used herein, the term "carbohydrates" includes, but is not limited to, disaccharides, trisaccharides, oligosaccharides and their corresponding sugar alcohols, polyhydroxy compounds, such as carbohydrate derivatives and chemically modified carbohydrates, hydroxyethyl starch and sugar copolymers (Ficoll). Both D and L forms of the

carbohydrates may be used. When the biological substance is used in a pharmaceutical preparation, the carbohydrate selected is physiologically acceptable. The carbohydrate may be non-reducing or reducing. As described in the examples presented herein, it has now been found that, surprisingly, non-reducing carbohydrates can hydrolyze to form reducing carbohydrates upon storage in dried compositions and initiate the formation of Schiff's bases with the substances stored therein. This condensation results in the formation of water which plasticizes the system, resulting in increased mobility of the chemical entities, thus enhancing the rate of the degradative reactions, including the Maillard reaction. Thus, the present invention is particularly useful with non-reducing carbohydrates.

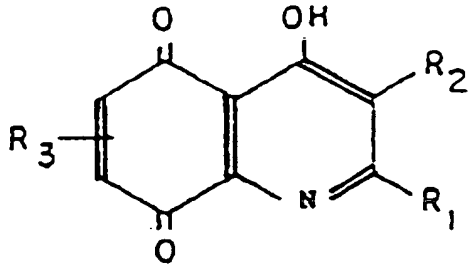
Reducing carbohydrates suitable for use in the present invention are those known in the art and include, but are not limited to, glucose, maltose, lactose, maltulose, iso-maltulose and lactulose.

Non-reducing carbohydrates include, but are not limited to, non-reducing glycosides of polyhydroxy compounds selected from sugar alcohols and other straight chain polyalcohols. Other useful carbohydrates include raffinose, stachyose, melezitose, dextran and sugar alcohols. The sugar alcohol glycosides are preferably monoglycosides, in particular the compounds obtained by reduction of disaccharides such as lactose, maltose, lactulose and maltulose. The glycosidic group is preferably a glucoside or a galactoside and the sugar alcohol is preferably sorbitol (glucitol). Particularly preferred carbohydrates are maltitol (4-O-β-D-glucopyranosyl-D-glucitol), lactitol (4-O-β-D-galactopyranosyl-D-glucitol), iso-maltulose, palatinit and its constituent isomers (GPS, α-D-glucopyranosyl-1→6-sorbitol and GPM, α-D-glucopyranosyl-1→6-

mannitol), the sugar alcohol derived from isomaltulose (palatinose) (6-α-D-glucopyranosyl-mannitol and 6-α-D-glucopyranosyl-sorbitol), sucrose and their sugar alcohols.

The inhibitor of the Maillard reaction can be any known in the art. When the biological substance is used in a pharmaceutical preparation, the inhibitor selected is physiologically acceptable. The inhibitor is present in an amount sufficient to prevent, or substantially prevent, condensation of amino groups and reactive carbonyl groups. Typically, the amino groups are present on the biological substance and the carbonyl groups are present on the carbohydrate. However, the amino and carbonyl groups may be intramolecular within either the biological substance or the carbohydrate. Various classes of compounds are known to exhibit an inhibiting effect on the Maillard reaction and hence to be of use in the compositions described herein. These compounds are generally either competitive or noncompetitive inhibitors. Competitive inhibitors include, but are not limited to, amino acid residues (both D and L), combinations of amino acid residues and peptides. Particularly preferred are lysine, arginine, histidine and tryptophan. Lysine and arginine are the most effective. There are many known noncompetitive inhibitors. These include, but are not limited to, aminoguanidine derivatives, amphotericin B and the following. EP-A-O 433 679 describes 4-hydroxy-5,8-dioxoquinoline derivatives of the following formulae.

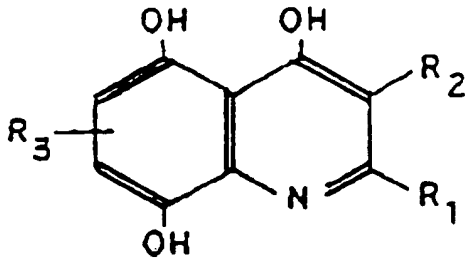
(a) a 4-hydroxy-5,8-dioxoquinoline derivative of the formula (I) or a salt thereof:



(I)

wherein R_1 and R_2 are each hydrogen, methyl, trifluoromethyl, carboxy, methoxycarbonyl or ethoxycarbonyl, and R_3 is hydrogen or hydroxy;

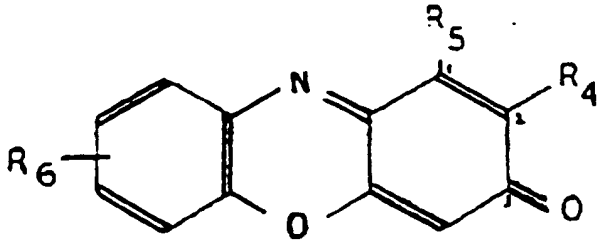
(b) a 4,5,8-trihydroxyquinoline derivative of the formula (II) or a salt thereof:



(II)

wherein R_1, R_2 and R_3 are as defined herein;

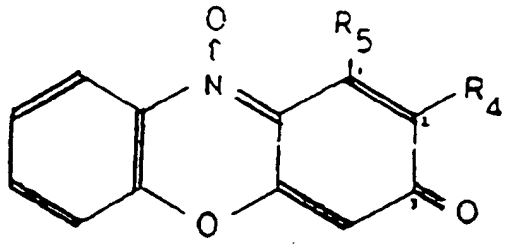
(c) a 3-oxophenoxazine derivative of the formula (III) or a salt thereof:



(III)

wherein R_4 and R_5 are each hydrogen or form by incorporation of C_1 and C_2 carbon atoms a condensed [2,1-b] pyridine ring optionally substituted with a hydroxyl group and/or a carboxyl group, and R_6 is hydrogen or hydroxy; or

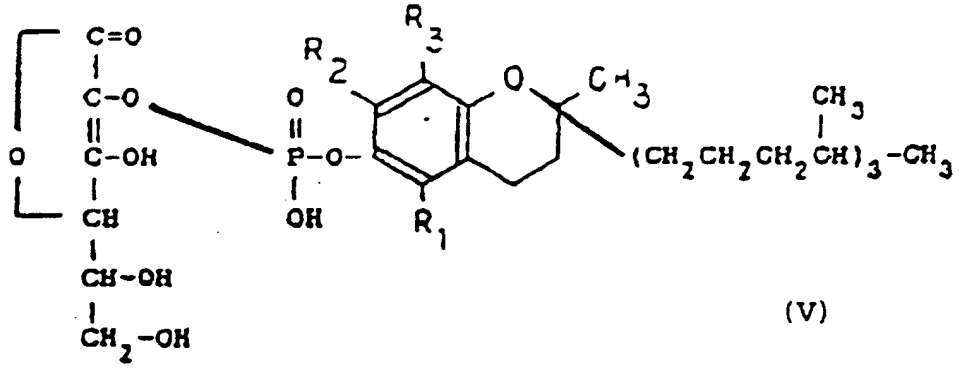
(d) a 3-oxophenoxazine N-oxide of the formula (IV) or a salt thereof:



(IV)

wherein R_4, R_5 and R_6 are as defined herein.

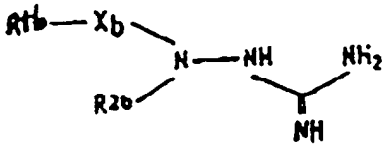
EP-A-0 430 045 similarly discloses ascorbic acid tocopheryl phosphate diesters having Maillard reaction-inhibiting action, namely compounds of the formula:



(V)

wherein R_1, R_2 and R_3 independently of one another denote hydrogen or methyl, or a salt thereof.

Also EP-A-0 325 936 describes aminoguanidine derivatives of the general formula:



(VI)

(wherein R^{1b} represents carbocyclic or heterocyclic ring substituted or unsubstituted by from 1 to 3 group(s) selected from halogen atom, alkyl or alkoxy group of from 1 to 4 carbon atom(s), nitro group, phenoxy group, amino group, hydroxy group and acylamino a group of from 2 to 4 carbon atom(s), X_b

represents single-bond, alkylene group of from 1 to 4 carbon atom(s) or alkenylene group of from 2 to 4 carbon atoms, or R^{1b} together with X_b represents alkyl group of from 1 to 4 carbon atom(s), R^{2b} represents hydrogen atom, alkyl group of from 1 to 4 carbon atom(s) or phenyl group substituted or unsubstituted by from 1 to 3 group(s) selected from halogen atom, alkyl or alkoxy group of from 1 to 14 carbon atom(s), hydroxy and nitro group, or an acid addition salt thereof.

In one preferred embodiment, the method of drying is freeze drying and the Maillard inhibitor is a competitive inhibitor. In another preferred embodiment, the method of drying is freeze drying and the Maillard inhibitor is a noncompetitive inhibitor. In another preferred embodiment, the method of drying is at ambient or elevated temperatures and the Maillard inhibitor is a competitive inhibitor. In another preferred embodiment, the method of drying is at ambient or elevated temperatures and the Maillard inhibitor is a noncompetitive inhibitor.

The invention also encompasses methods of increasing the "shelf-life" or storage stability of dried biological substances stored at elevated temperatures. Increased storage stability is determined by recovery of biological activity in accelerated aging trials. The methods include drying, or otherwise incorporating, at least one biological substance in the presence of at least one carbohydrate excipient in an amount sufficient to stabilize the biological substance, and at least one inhibitor of the Maillard reaction. Other suitable buffers, salts, cofactors, etc., may be added to the composition. The composition may be stored at any suitable temperature. Preferably, the compositions are stored at 0°C to 80°C. More preferably, the compositions are stored at

20°C-60°C. Most preferably, the compositions are stored at above ambient temperatures.

The invention also encompasses compositions with increased storage stability comprising a biological substance, a carbohydrate in an amount sufficient to stabilize the dried biological substance and an inhibitor of the Maillard reaction wherein the carbohydrate is present in an amount sufficient to stabilize the biological substances upon drying and the inhibitor is present in an amount sufficient to substantially prevent condensation of amino groups on the biological substances and reactive carbonyl groups therein, or on the carbohydrate stabilizing excipient, or generated therefrom. Preferably, the compositions have increased storage stability at 0°C-250°C. More preferably, the compositions have increased storage stability at 20°C-50°C. Most preferably, the compositions have increased stability at above ambient temperatures.

Preferably, the composition is almost completely dry so as to increase stability of the molecule by decreasing the Maillard reaction during storage. Some water or other aqueous solvent may remain in the composition but preferably, not more than 20%. As used herein, "dry," "dried," and "substantially dried" encompass those compositions with from about 0-20% water. Preferably, less than about 20% water is present, more preferably less than about 10% water is present, and most preferably less than about 5% water is present.

The compositions may be obtained by any of the methods described above and may contain any of the components described above. Although actual weights and percentages of the various components of the compositions are not given, they are within the skill of one in the art to determine without undue experimentation given the disclosure herein.

Example 1

Trehalose stabilization of biomolecules

It has previously been shown that antibodies, air-dried in the presence of trehalose, are undamaged, and full biological activity is recovered on rehydration, even after several years storage at room temperature or 37°C. Roser (1991); Blakely et al. (1990); and Colaco et al. (1990). Similar results were obtained with a variety of enzymes, hormones and blood coagulation factors, suggesting that this process may be generally applicable to biological substances. Roser (1991); Roser and Colaco (1993); Blakely et al. (1990); Colaco et al. (1990); and Colaco et al. (1992). As a stringent test of this technology to preserve labile biological substances, the enzymes used in molecular biology, which are notoriously fragile and thus usually transported and stored at or below -20°C, were studied in detail. It has previously been shown that both restriction endonucleases and DNA modifying enzymes can be dried from trehalose solutions at ambient temperatures without loss of activity. Furthermore, these dried enzymes show stability on storage for extended periods even at elevated temperatures. Colaco et al (1992).

In order to determine the ability of carbohydrates other than trehalose to preserve activity in proteins, an accelerated aging study was performed with the restriction enzyme *Pst*I vacuum-dried with supplemental heating to a residual moisture content of 2.6-3.6% in a number of carbohydrate excipients and stored for one month at either 37°C, 55°C or 70°C.

The results obtained are presented in Table 1 and Figure 1. Five units of fresh enzyme control (track 1) was compared, for the ability to cut bacteriophage λ DNA, with 2.5 unit (Fig. 1. even

numbered tracks) or 5 unit (Fig. 1, odd numbered tracks) of enzyme dried using various carbohydrate excipients and stored for 35 days at 37°C (Fig. 1, top panel), 55°C (Fig. 1, middle panel) and 70°C (Fig. 1, bottom panel). The carbohydrate excipients were used Glucopyranosyl-mannitol (Fig. 1, tracks 3 & 4) or sorbitol (Fig. 1, tracks 5 & 6), reduced isomaltose (Fig. 1, tracks 7 & 8), sucrose (Fig. 1, tracks 9 & 10), maltose (Fig. 1, tracks 11 & 12) and trehalose (Fig. 1, tracks 13 & 14). As can be seen from Fig. 1, only trehalose shows any stabilizing effects at the two higher temperatures studied.

Although some of the carbohydrate excipients stabilized the enzyme during drying and on storage at 37°C (Fig. 1, top panel, Table 1), only the samples stabilized using trehalose retained activity when stored at either 55°C or 70°C (Fig. 1, middle and bottom panels, Table 1). These results have now been correlated with real-time data and emphasize the finding that, with respect to long-term stability, trehalose is a better stabilizing excipient than the other carbohydrates tested under identical drying and storage conditions (Table 1). All the monosaccharides were ineffective, whether reducing or non-reducing, as were polymers such as inulin, ficoll and dextran (Table 1). Reducing sugars, such as lactose and maltose, failed within a month at the lowest temperature studied, 37°C, as did the non-reducing disaccharide sucrose (Table 1). The chemically more stable non-reducing sugars, the sugar alcohols, showed better stabilities than their reducing counterparts, but still failed within a month at 55°C (Table 1).

TABLE 1: STABILITY OF PST I DRIED IN VARIOUS CARBOHYDRATE EXCIPIENTS

CARBOHYDRATE	CHEMICAL NAME	RED SUGAR	TEMP °C	TIME days	ACTIVITY
MONOSACCHARIDES AND ALCOHOLS					
Glucose	α-D-glucopyranose	+	37°C	1	+
			"	14	-
Sorbitol	sugar alcohol of glucose	-	"	14	+
			"	35	+
			"	70	-
Galactose	α-D-galactopyranose	+	"	1	-
Galactitol	sugar alcohol of galactose	-	"	1	-
Mannose	α-D-mannopyranose	+	"	1	-
Mannitol	sugar alcohol of mannose	-	"	1	-
DISACCHARIDES					
Trehalose	α-D-glucopyranosyl-α-D-glucopyranoside	-	"	98	+ + +
			55°C	70	+ + +
			70°C	35	+ + +
Maltose	4-O-α-D-glucopyranosyl-D-glucose	+	"	14	+ +
			"	7	-

TABLE 1 (cont'd)

CARBOHYDRATE	CHEMICAL NAME	RED SUGAR	TEMP °C	TIME days	ACTIVITY
Maltotriose	O-α-D-glucopyranosyl(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucose	+	"	14	-
Lactose	4-O-β-D-galactopyranosyl-D-glucopyranose	+	"	14	-
Lactulose	4-O-β-D-galactopyranosyl-D-fructose	+	"	14	+
				35	-
Sucrose	β-D-fructofuranosyl-α-D-glucopyranoside	-	37°C	14	+ +
			"	35	-
POLYMERS					
Inulin	Polymer of 1-O-β-D-fructofuranosyl-D-fructose	-	"	7	-
Dextran	Polymer of α-(1→6)-D-glucopyranose (1→3, 1→4 branch)	+	"	7	-
Ficoll	Polymer of β-D-fructofuranosyl-α-D-glucopyranose	-	"	7	+

Quantitation of activity
- no detectable activity
+ some activity (10-20% of titre)
+ + partial activity (25-40% of titre)
+ + + full activity

Reducing properties
+ reducing sugar
- non-reducing sugar

Example 2

Non-Enzymatic browning of dried formulations during storage

It appears that the relative chemical stability and non-reducing nature of trehalose may be significant features in its mechanism of action, especially with regard to the long term stability observed at high temperatures. This was first suggested by a surprising feature observed in the accelerated ageing trial described in Fig. 1 above. As shown in Fig. 2, non-enzymatic browning was observed in the samples used in the accelerated ageing study reported in Fig. 1 after two weeks storage at 37°C (top panel), 55°C (middle panel) and 70°C (bottom panel). The carbohydrate excipients used were trehalose (row 1), sucrose (row 2), maltose (row 3), reduced iso-maltulose (row 4), glucopyranosyl-sorbitol (row 5) and glucopyranosyl-mannitol (row 6).

The results obtained indicate that the development of a brown coloration developed in a number of the sample wells at all three temperatures after just two weeks of storage of the sample. Furthermore, the extent of the coloration appeared to correlate with the reduction in enzymatic activity in these samples. Increasing coloration was observed in the samples stored at higher temperatures (Fig. 2), which also showed the greatest loss of activity (Fig. 1). This coloration was highly reminiscent of the non-enzymatic browning seen during the processing and storage of food products. This non-enzymatic browning is the result of the spontaneous reaction between the reducing sugars and proteins that are natural constituents of these foodstuffs, and has been widely studied in food chemistry under the umbrella of the so-called Maillard reaction.

Example 3

Evidence for Maillard reactions in dried formulations

Studies were undertaken to correlate changes in biological activity with the development of brown pigments, as observed in the accelerated ageing studies on restriction enzymes (Fig. 2). These studies were carried out on samples of the enzyme alkaline phosphates which were dried, under the conditions described in Example 1 above, from solutions containing glucose, fructose, maltose or trehalose and stored for various periods at 55°C, before enzymatic activity was reassayed. Residual activity was determined in samples of alkaline phosphatase dried in the presence of various carbohydrate excipients and assayed calorimetrically after storage at 55°C.

Development of brown coloration assayed by absorbance between 277-290nm was compared with loss of enzymic activity in samples of alkaline phosphatase dried in fructose and glucose after storage at 55°C. Figure 5 shows the results obtained where color development was measured at O.D. 277-290; (·) glucose, (+) fructose. Enzymic activity (O.D. 405); and depicted as follows: (*) glucose, (□) fructose. The production of melanoid pigments occurred later than the loss of enzymatic activity assayed calorimetrically (Fig. 4). This is consistent with the fact that the generation of brown melanoid pigments occurs in the terminal stages of the Maillard reaction and thus cannot be used to predict enzyme inactivation due to the early reactions of the cascade (Fig. 3). Similarly, analysis of the samples by SDS-PAGE showed a complex pattern of protein breakdown and cross-linking in all samples, except those dried in trehalose, and the complexity of these patterns precludes the use of this technique in determining the

extent of protein modification by the Maillard reaction.

A surprising result was obtained on analysis of the residual sugar contents of the samples in the studies described above. When dried in glucose or fructose, only the individual sugars were detectable in the samples immediately post-drying. However, on loss of enzymatic activity after high temperature storage, these samples were found to contain mixtures of the two sugars. These results are presented in Table 2.

A similar isomerization was observed in the samples found to contain a mixture of glucose and maltose immediately post-drying, due to partial hydrolysis of the maltose. On loss of activity after high temperature storage, these samples were found to contain a mixture of both glucose and fructose, as well as maltose (Table 2). The absence of any mannose production detected in this non-enzymatic isomerization is indicative of chemical reaction pathway involving a common Schiff's base intermediate, similar to the formation of osazones in the Fisher reaction.

TABLE 2: HPLC ANALYSIS OF CARBOHYDRATES IN FORMULATIONS DRIED IN VARIOUS EXCIPIENTS

CARBOHYDRATE EXCIPIENT USED IN TWO FORMULATIONS	POST DRYING ACTIVITY	POST DRYING SUGAR ANALYSIS	ACTIVITY AFTER 2 WEEKS	HPLC ANALYSIS AFTER 2 WEEKS STORAGE
1. Glucose Sample A	+	Glucose	-	Glucose + Fructose
2. Glucose Sample B	+	Glucose	-	Glucose + Fructose
3. Sorbitol Sample A	+ +	Sorbitol	-	Sorbitol
4. Sorbitol Sample B	+ +	Sorbitol	-	Sorbitol
5. Fructose Sample A	+	Fructose	-	Fructose + Glucose
6. Fructose Sample B	+	Fructose	-	Fructose + Glucose
7. Maltose Sample A	+ + +	Maltose + Glucose	-	Maltose + Glucose + Fructose
8. Maltose Sample B	+ + +	Maltose	+/-	Maltose + Glucose + "Others"
9. Sucrose Sample A	+ + + + +	Sucrose	+ + +	Sucrose
10. Sucrose Sample B	+ + + + +	Sucrose	+ +	Sucrose
11. Trehalose Sample A	+ + + + +	Trehalose	+ + + + +	Trehalose
12. Trehalose Sample B	+ + + + +	Trehalose	+ + + + +	Trehalose

Example 4

Maillard-Related modifications of Glucagon

To exemplify the generality of these chemical modifications of proteins by carbohydrate excipients, a relevant pharmaceutical model system was studied. The protein modifications of a therapeutic peptide, glucagon, dried from solution for 18 hr under a vacuum of 30 milliTorr, with a shelf-temperature rising from 25 to 42°C, were studied. Formulations containing various carbohydrate excipients were analyzed by reverse-phase HPLC analysis. The results obtained from a comparison of glucose and sucrose stored for varying times are presented in Fig. 5.

The results depicted in Figure 5 show that greater than 98% of untreated glucagon runs as a single monomer with a retention time of about 20 minutes (Fig. 5A). Immediately after drying in glucose, there was a single peak of glycated glucagon (shaded) running immediately ahead of the monomer peak (Fig. 5B). Within 4 days at 60°C in glucose the majority of the glucagon was destroyed by glycation and cross-linking (shaded, Fig. 5C). After 2 weeks storage in sucrose, a peak of glycated glucagon runs immediately ahead of the main monomer peak (shaded, Fig. 5D) even though no such peak was evident in the sample after drying.

These results indicate that in the case of glucose (a reducing sugar), the Schiff's base is formed during drying whereas in the case of sucrose (a non-reducing sugar), a Schiff's base is formed upon storage. As a non-reducing sugar, sucrose would not be expected to form a Schiff's base upon either drying or storage. This indicates that the Maillard reaction occurs with both reducing and non-reducing sugars, and thus the present invention is suitable for use with both reducing and non-reducing sugars. These results further indicate that, in the case of sucrose as an

excipient, a reducing sugar is generated by hydrolysis that may be catalyzed by the substance being stored. Furthermore, the Schiff's base formation by the reducing sugar generated releases water which not only plasticizes the system but directly accelerates both further hydrolysis reactions and the Maillard cascade (see Example 5, below). Thus, in light of the results presented herein, the use of an inhibitor of the Maillard reaction is particularly useful in conjunction with a non-reducing sugar in dry storage of biological substances.

EXAMPLE 5

The Effect of Water on Storage Stability

To investigate directly the effect of residual moisture on chemical reactivity, a model system containing lysine with two non-reducing excipients trehalose and sorbitol, dried and stored at 3 different defined residual water contents, was used. Chemical reactivity was ensured by spiking the drying mixtures with a 5% trace of glucose, and its reaction with the lysine was measured by the quantitation of the glucose remaining after storage at 50°C.

Figure 6 shows the results obtained where solutions of 10% w/v lysine and 5% glucose in 85% trehalose (a) or 85% sorbitol (b) in water were freeze-dried with primary drying at -50°C for 48 hr and secondary drying for a further 24 hr at 20°C. The desired water content was achieved by storage at 20°C over anhydrous P_2O_5 followed by exposure to a saturated water vapor atmosphere for either O hr, 8 hr or 25 hr. The actual final water content of the samples was determined by thermogravimetry using a Kahn microbalance. Figure 6 depicts the results as follows: a) (□) 4.59% water, (◇) 15.09% water, (x) 22.89% water; and b) (□) 5.42% water, (◇) 13.12% water, (x) 23.62% water.

The results obtained indicate that in the trehalose samples, chemical reactivity was reduced as the residual water content decreased, and essentially no reactivity was observed at a water content of around 5% (Fig. 6a). In contrast, in the sorbitol samples, the chemical reactivity was accentuated under the driest conditions (Fig. 6b). The results show that Maillard-type reactions are extremely important during the storage of dried products, as these reactions are driven to completion in systems whether water activity is limiting.

EXAMPLE 6

The Effect of a Noncompetitive Inhibitor of the Maillard Reaction

Bovine serum albumin was dried from solutions containing reducing sugars and incubated at 55°C for 3 weeks, in the presence or absence of various concentrations of the Maillard reaction inhibitor aminoguanidine. The extent of the Maillard reaction was quantified spectrophotometrically at 277-290 nm by the formation of brown color.

The results obtained are shown in Table 3.

Table 3

Sugar (25 mM)	Potential Inhibitor	Ratio Sugar: Inhibitor (molar basis)	Absorbance extent of browning
Glucose	None		0.709
Glucose	aminoguanidine	3:1	0.603
Glucose	aminoguanidine	2:1	0.205
Glucose	aminoguanidine	1:1	0.212
Glucose	aminoguanidine	1:2	0.211
Glucose	aminoguanidine	1:3	0.154
None	aminoguanidine	1:3	0.186
Fructose	None		2.5
Fructose	aminoguanidine	1:3	0.173

The results obtained indicate that the addition of a Maillard reaction inhibitor to a protein formulation being dried, prevents the degradation of the dried protein product during storage at elevated temperatures.

EXAMPLE 7

The effect of competitive inhibitors of the Maillard reaction

In order to determine the effect of competitive inhibitors on reducing the effects of the Maillard reaction upon storage of dried compositions, the following experiment was performed. The results are presented in Figure 7.

Alkaline phosphatase (0.25 mg/ml) was dried under vacuum (80 microns) at ambient temperature for 16 hours from a formulation containing 15% glucose as the carbohydrate excipient with either 0, 7.5, 15 and 25% lysine added as a competitive Maillard reaction inhibitor. The samples were stored for four days at 55°C and assayed by running on a 10% polyacrylamide gel and stained for enzymatic activity using α-naphthyl acid phosphate and Fast Blue BB (1 mg/ml and 1.33 mg/ml, respectively, in 0.38 M Tris-HCl (pH 10.3) containing 0.5 mM $MgCl_2$) (Fig. 7, tracks 1-4, respectively). Controls run were enzyme in aqueous buffer and enzyme dried in trehalose (Fig. 7, lanes 8-9, respectively). Although only partial activity was recovered in the glucose plus lysine samples, these showed increased activity recovered with increasing concentration of added lysine. In the sample with glucose but with no added lysine, no enzymatic activity was recovered.

Thus, a competitive inhibitor of the Maillard reaction, when dried with a mixture of protein and carbohydrate, increases the stability of

the protein during drying and storage of the dried product.

In conclusion, our results suggest that the Maillard reaction is an extremely important factor in determining the long term storage stability of dry protein formulations containing carbohydrates as stabilizing excipients. While not being bound by any one theory, this may be due to both the shift in the equilibrium of the initial amino-carbonyl condensation reaction towards the formation of the Schiff's base by the removal of water during drying and the acceleration of the subsequent reactions at low water activities. Lendl and Schleicher (1990) and Nursten (1986).

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of understanding it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

CLAIMS

1. A method of increasing stability of biological substances during drying comprising adding to an aqueous solution or suspension of the substances and a carbohydrate excipient in an amount sufficient to stabilize the substances, inhibitors of the Maillard reaction in an amount effective to substantially prevent condensation of amino groups and reactive carbonyl groups and drying the resulting composition.

2. The method according to claim 1 wherein the biological substances are selected from the group consisting of subcellular compositions, cells and viruses.

3. The method according to claim 1 wherein the biological substances are selected from the group consisting of molecules containing free amino, imino and guanidino side chains.

4. The method according to claim 3 wherein the biological substances are selected from the group consisting of pharmaceutical agents, lipids, organics, peptides, proteins, hormones, saccharides, oligosaccharides, synthetic peptides, D and L amino acid polymers, nucleic acids, protein nucleic acid hybrids and small molecules.

5. The method according to claim 4 wherein the proteins are selected from the group consisting of enzymes, biopharmaceuticals, growth hormones, growth factors, monoclonal antibodies and cytokines.

6. The method according to claim 1 wherein the method of drying is selected from the group consisting of freeze drying, spray drying, fluidized

bed drying, drum drying, drying at ambient temperature and atmosphere pressure, drying at ambient temperature and decreased pressure, drying at elevated temperatures and atmospheric pressure and drying at elevated temperatures and decreased pressure.

7. The method according to claim 1 wherein the carbohydrate is selected from the group consisting of disaccharides, trisaccharides, oligosaccharides and sugar alcohols thereof.

8. The method according to claim 7 wherein the carbohydrate is reducing.

9. The method according to claim 8 wherein the carbohydrate is selected from the group consisting of glucose, maltose, lactose, maltulose, iso-maltulose and lactulose.

10. The method according to claim 7 wherein the carbohydrate is nonreducing.

11. The method according to claim 10 wherein the carbohydrate is selected from the group consisting of non-reducing glycosides of polyhydroxy compounds selected from sugar alcohols and other straight chain polyalcohols.

12. The method according to claim 10 wherein the carbohydrate is selected from the group consisting of raffinose, stachyose, melezitose, dextran and sugar alcohols.

13. The method according to claim 10 wherein the carbohydrate is selected from the group consisting of maltitol (4-O-β-D-glucopyranosyl-D-glucitol), lactitol (4-O-β-D-galactopyranosyl-D-

glucitol), iso-maltulose, palatinit and its constituent isomers (GPS, α-D-glucopyranosyl-1→ 6-sorbitol and GPM, α-D-glucopyranosyl-1→6-mannitol), the sugar alcohol derived from isomaltulose (palatinose) (6-α-D-glucopyranosyl-mannitol and 6-α-D-glucopyranosyl-sorbitol), sucrose and the corresponding sugar alcohols.

14. The method according to claim 7 wherein the carbohydrate is physiologically acceptable.

15. The method according to claim 1 wherein the inhibitor is competitive.

16. The method according to claim 15 wherein the inhibitor is a molecule with at least one primary, secondary or tertiary amine group.

17. The method according to claim 1 wherein the inhibitor is noncompetitive.

18. The method according to claim 17 wherein the inhibitor is selected from the group consisting of aminoguanidine derivatives, amphotericin B, 4-hydroxy-5,8-dioxoquinoline derivatives, 4,5,8-trihydroxyquinoline derivatives, 3-oxophenoxazine derivatives, 3-oxophenoxazine N-oxide, ascorbic acid tocopheryl phosphate diesters and salts thereof.

19. The method according to claim 1 wherein the method of drying is freeze drying and the inhibitor is a competitive inhibitor.

20. The method according to claim 1 wherein the method of drying is freeze drying and the inhibitor is a noncompetitive inhibitor.

21. The method according to claim 1 wherein the method of drying is other than freeze drying and the inhibitor is a competitive inhibitor.

22. The method according to claim 1 wherein the method of drying is other than freeze drying and the inhibitor is noncompetitive.

23. A method of increasing storage stability of biological substances comprising adding a carbohydrate excipient in an amount effective to increase stability of the substance adding inhibitors of the Maillard reaction in an amount effective to substantially prevent condensation of amino groups and reactive carbonyl groups, drying the resulting composition and storing the composition.

24. The method according to claim 23 wherein the biological substances are selected from the group consisting of subcellular fragments, cells and viruses.

25. The method according to claim 23 wherein the biological substances are selected from the group consisting of molecules containing free amino, imino and guanidino side chains.

26. The method according to claim 25 wherein the biological substances are selected from the group consisting of pharmaceutical agents, lipids, organics, proteins, hormones, saccharides, oligosaccharides, synthetic peptides, D and L amino acid polymers, nucleic acids, protein nucleic acid hybrids and small molecules.

27. The method according to claim 26 wherein the proteins are selected from the group

consisting of enzymes, biopharmaceuticals, growth hormones, growth factors, monoclonal antibodies and cytokines.

28. The method according to claim 23 wherein the method of drying is selected from the group consisting of freeze drying, spray drying, fluidized bed drying, drum drying, drying at ambient temperature and atmosphere pressure, drying at ambient temperature and decreased pressure, drying at elevated temperatures and atmospheric pressure and drying at elevated temperatures and decreased pressure.

29. The method according to claim 23 wherein the carbohydrate is selected from the group consisting of disaccharides, trisaccharides, oligosaccharides and sugar alcohols thereof.

30. The method according to claim 29 wherein the carbohydrate is reducing.

31. The method according to claim 30 wherein the carbohydrate is selected from the group consisting of glucose, maltose, lactose, maltulose, iso-maltulose and lactulose.

32. The method according to claim 29 wherein the carbohydrate is nonreducing.

33. The method according to claim 32 wherein the carbohydrate is selected from the group consisting of non-reducing glycosides of polyhydroxy compounds selected from sugar alcohols and other straight chain polyalcohols.

34. The method according to claim 32 wherein the carbohydrate is selected from the group

consisting of raffinose, stachyose, melezitose, dextran and sugar alcohols.

35. The method according to claim 32 wherein the carbohydrate is selected from the group consisting of maltitol (4-O-β-D-glucopyranosyl-D-glucitol), lactitol (4-O-β-D-galactopyranosyl-D-glucitol), iso-maltulose, palatinit and its constituent isomers (GPS, α-D-glucopyranosyl-1→ 6-sorbitol and GPM, α-D-glucopyranosyl-1→6-mannitol), the sugar alcohol derived from isomaltulose (palatinose) (6-α-D-glucopyranosyl-mannitol and 6-α-D-glucopyranosyl-sorbitol), sucrose and the corresponding sugar alcohols.

36. The method according to claim 31 wherein the carbohydrate is physiologically acceptable.

37. The method according to claim 23 wherein the inhibitor is competitive.

38. The method according to claim 37 wherein the inhibitor is a molecule with at least one primary, secondary or tertiary amine group.

39. The method according to claim 38 wherein the inhibitor is selected from the group consisting of at least one amino acid residue, a mixture of amino acid residues or short peptides comprised of amino acid residues.

40. The method according to claim 23 wherein the inhibitor is noncompetitive.

41. The method according to claim 40 wherein the inhibitor is selected from the group

consisting of aminoguanidine derivatives, amphotericin B, 4-hydroxy-5,8-dioxoquinoline derivatives, 4,5,8-trihydroxyquinoline derivatives, 3-oxophenoxazine derivatives, 3-oxophenoxazine N-oxide, ascorbic acid tocopheryl phosphate diesters and salts thereof.

42. The method according to claim 23 wherein storage is at 0°C-80°C.

43. The method according to claim 23 wherein storage is at 0°-65°C.

44. The method according to claim 23 wherein storage is at 10°C-60°C.

45. The method according to claim 23 wherein storage is at 20°-50°C.

46. The method according to claim 23 wherein storage is at above ambient temperature.

47. A method of increasing storage stability of dried biological substances comprising combining the biological substance, a carbohydrate excipient in an amount effective to increase stability of the substance adding inhibitors of the Maillard reaction in an amount effective to substantially prevent condensation of amino groups and reactive carbonyl groups, and storing the composition.

48. The method according to claim 47 wherein the biological substances are selected from the group consisting of subcellular fragments, cells and viruses.

49. The method according to claim 47 wherein the biological substances are selected from

the group consisting of molecules containing free amino, imino and guanidino side chains.

50. The method according to claim 49 wherein the biological substances are selected from the group consisting of pharmaceutical agents, lipids, organics, peptides, proteins, hormones, saccharides, oligosaccharides, synthetic peptides, D and L amino acid polymers, nucleic acids, protein nucleic acid hybrids and small molecules.

51. The method according to claim 50 wherein the proteins are selected from the group consisting of enzymes, biopharmaceuticals, growth hormones, growth factors, monoclonal antibodies and cytokines.

52. The method according to claim 47 wherein the method of drying is selected from the group consisting of freeze drying, spray drying, fluidized bed drying, drum drying, drying at ambient temperature and atmosphere pressure, drying at ambient temperature and decreased pressure, drying at elevated temperatures and atmospheric pressure and drying at elevated temperatures and decreased pressure.

53. The method according to claim 47 wherein the carbohydrate is selected from the group consisting of disaccharides, trisaccharides, oligosaccharides and sugar alcohols thereof.

54. The method according to claim 53 wherein the carbohydrate is reducing.

55. The method according to claim 54 wherein the carbohydrate is selected from the group

consisting of glucose, maltose, lactose, maltulose, iso-maltulose and lactulose.

56. The method according to claim 53 wherein the carbohydrate is nonreducing.

57. The method according to claim 56 wherein the carbohydrate is selected from the group consisting of non-reducing glycosides of polyhydroxy compounds selected from sugar alcohols and other straight chain polyalcohols.

58. The method according to claim 56 wherein the carbohydrate is selected from the group consisting of raffinose, stachyose, melezitose, dextran and sugar alcohols.

59. The method according to claim 56 wherein the carbohydrate is selected from the group consisting of maltitol (4-O-β-D-glucopyranosyl-D-glucitol), lactitol (4-O-β-D-galactopyranosyl-D-glucitol), iso-maltulose, palatinit and its constituent isomers (GPS, α-D-glucopyranosyl-1→ 6-sorbitol and GPM, α-D-glucopyranosyl-1→6-mannitol), the sugar alcohol derived from isomaltulose (palatinose) (6-α-D-glucopyranosyl-mannitol and 6-α-D-glucopyranosyl-sorbitol), sucrose and the corresponding sugar alcohols.

60. The method according to claim 53 wherein the carbohydrate is physiologically acceptable.

61. The method according to claim 47 wherein the inhibitor is competitive.

62. The method according to claim 61 wherein the inhibitor is a molecule with at least one primary, secondary or tertiary amine group.

63. The method according to claim 62 wherein the inhibitor is selected from the group consisting of at least one amino acid residue, a mixture of amino acid residues or short peptides comprised of amino acid residues.

64. The method according to claim 47 wherein the inhibitor is noncompetitive.

65. The method according to claim 64 wherein the inhibitor is selected from the group consisting of aminoguanidine derivatives, amphotericin B, 4-hydroxy-5,8-dioxoquinoline derivatives, 4,5,8-trihydroxyquinoline derivatives, 3-oxophenoxazine derivatives, 3-oxophenoxazine N-oxide, ascorbic acid tocopheryl phosphate diesters and salts thereof.

66. The method according to claim 47 wherein storage is at 0°C-80°C.

67. The method according to claim 47 wherein storage is at 0°-65°C.

68. The method according to claim 47 wherein storage is at 10°C-60°C.

69. The method according to claim 47 wherein storage is at 20°-50°C.

70. The method according to claim 47 wherein storage is at above ambient temperature.

71. A substantially dry composition comprising at least one biological substance, at least one carbohydrate and an inhibitor of the Maillard reaction wherein the carbohydrate is present in an amount sufficient to stabilize the biological substance and the inhibitor is present in an amount sufficient to substantially prevent condensation of amino groups and reactive carbonyl groups.

72. The composition according to claim 71 wherein the biological substances are selected from the group consisting of subcellular fragments, cells and viruses.

73. The composition according to claim 71 wherein the biological substances are selected from the group consisting of molecules containing free amino, imino and guanidino side chains.

74. The composition according to claim 73 wherein the biological substances are selected from the group consisting of pharmaceutical agents, lipids, organics, proteins, peptides, hormones, saccharides, oligosaccharides, synthetic peptides, D and L amino acid polymers, nucleic acids, protein nucleic acid hybrids and small molecules.

75. The composition according to claim 74 wherein the proteins are selected from the group consisting of enzymes, biopharmaceuticals, growth hormones, growth factors, monoclonal antibodies and cytokines.

76. The composition according to claim 71 wherein the composition of drying is selected from the group consisting of freeze drying, spray drying, fluidized bed drying, drum drying, drying at ambient

temperature and atmosphere pressure, drying at ambient temperature and decreased pressure, drying at elevated temperatures and atmospheric pressure and drying at elevated temperatures and decreased pressure.

77. The composition according to claim 71 wherein the carbohydrate is selected from the group consisting of disaccharides, trisaccharides, oligosaccharides and sugar alcohols thereof.

78. The composition according to claim 77 wherein the carbohydrate is reducing.

79. The composition according to claim 78 wherein the carbohydrate is selected from the group consisting of glucose, maltose, lactose, maltulose, iso-maltulose and lactulose.

80. The composition according to claim 77 wherein the carbohydrate is nonreducing.

81. The composition according to claim 80 wherein the carbohydrate is selected from the group consisting of non-reducing glycosides of polyhydroxy compounds selected from sugar alcohols and other straight chain polyalcohols.

82. The composition according to claim 81 wherein the carbohydrate is selected from the group consisting of raffinose, stachyose, melezitose, dextran and sugar alcohols.

83. The composition according to claim 81 wherein the carbohydrate is selected from the group consisting of maltitol (4-O-β-D-glucopyranosyl-D-glucitol), lactitol (4-O-β-D-galactopyranosyl-D-glucitol), iso-maltulose, palatinit and its

constituent isomers (GPS, α-D-glucopyranosyl-1→ 6-sorbitol and GPM, α-D-glucopyranosyl-1→6-mannitol), the sugar alcohol derived from isomaltulose (palatinose) (6-α-D-glucopyranosyl-mannitol and 6-α-D-glucopyranosyl-sorbitol), sucrose and the corresponding sugar alcohols.

84. The composition according to claim 77 wherein the carbohydrate is physiologically acceptable.

85. The composition according to claim 71 wherein the inhibitor is competitive.

86. The composition according to claim 85 wherein the inhibitor is a molecule with at least one primary, secondary or tertiary amine group.

87. The composition according to claim 86 wherein the inhibitor is selected from the group consisting of at least one amino acid residue, a mixture of amino acid residues or short peptides comprised of amino acid residues.

88. The composition according to claim 71 wherein the inhibitor is noncompetitive.

89. The composition according to claim 88 wherein the inhibitor is selected from the group consisting of aminoguanidine derivatives, amphotericin B, 4-hydroxy-5,8-dioxoquinoline derivatives, 4,5,8-trihydroxyquinoline derivatives, 3-oxophenoxazine derivatives, 3-oxophenoxazine N-oxide, ascorbic acid tocopheryl phosphate diesters and salts thereof.

90. The composition according to claim 71 the biological substance is storage stable at 0°C-80°C.

91. The composition according to claim 71 wherein the biological substance is storage stable at 0°-65°C.

92. The composition according to claim 71 wherein the biological substance is storage stable at 10°C-60°C.

93. The composition according to claim 71 wherein the biological substance is storage stable at 20°-50°C.

94. The composition according to claim 71 wherein the biological substance is storage stable at above ambient temperature.

95. A composition for increasing storage stability of biological substances comprising at least one carbohydrate and an inhibitor of the Maillard reaction wherein the carbohydrate is present in an amount sufficient to stabilize the biological substance and the inhibitor is present in an amount sufficient to substantially prevent condensation of amino groups and reactive carbonyl groups.

FIG. 1

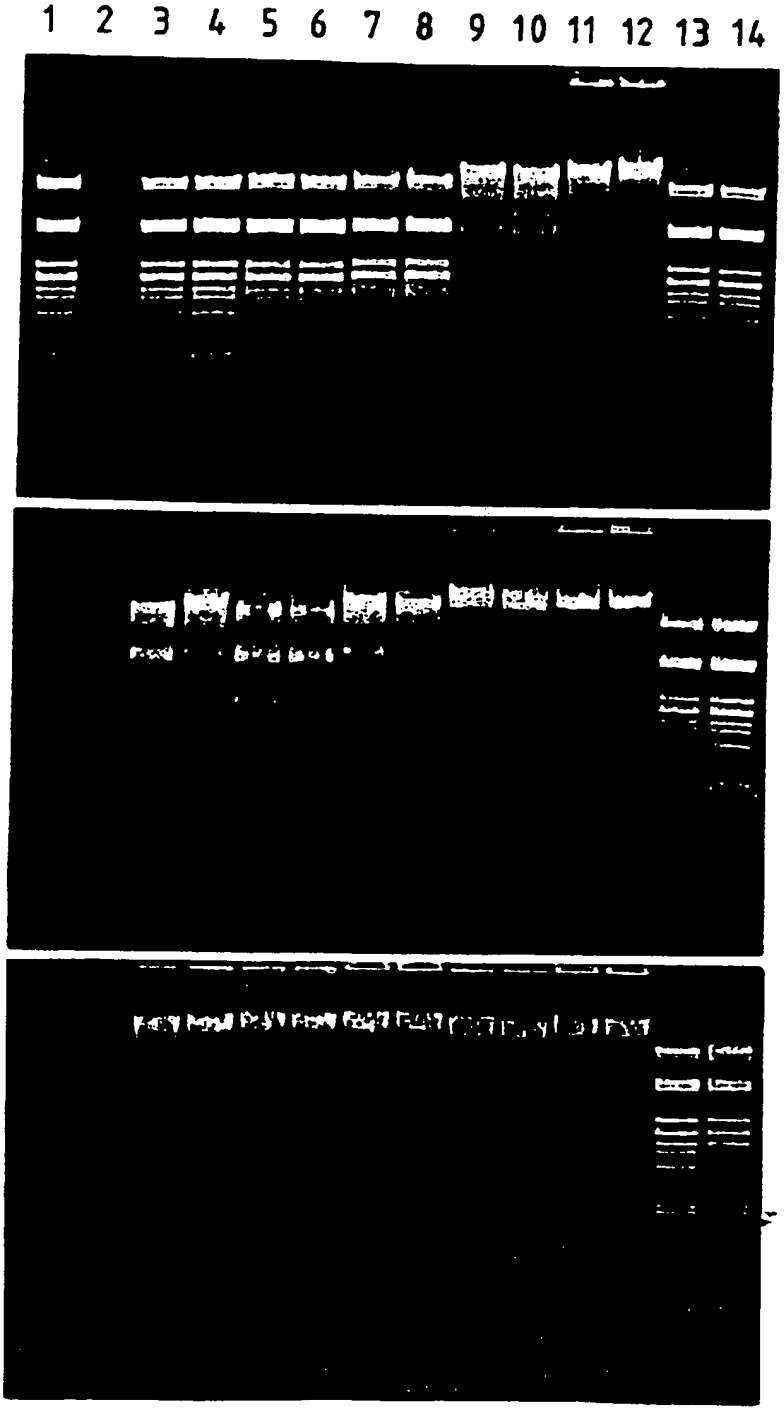


FIG. 2

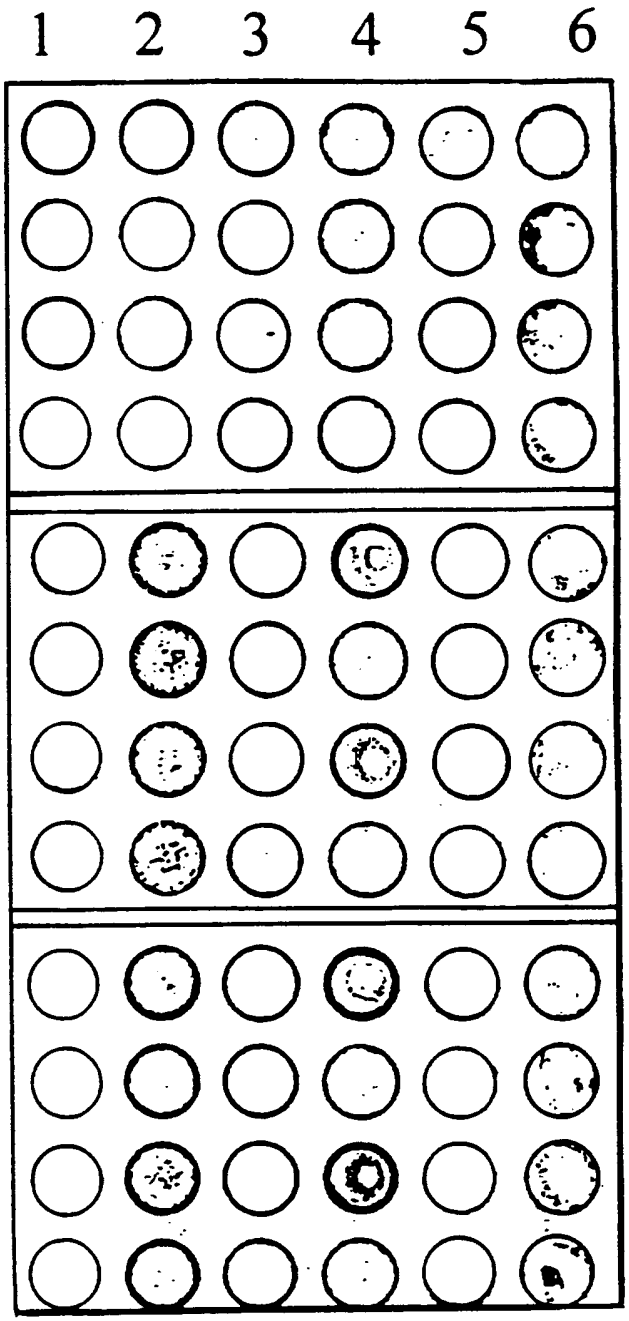


FIG. 3

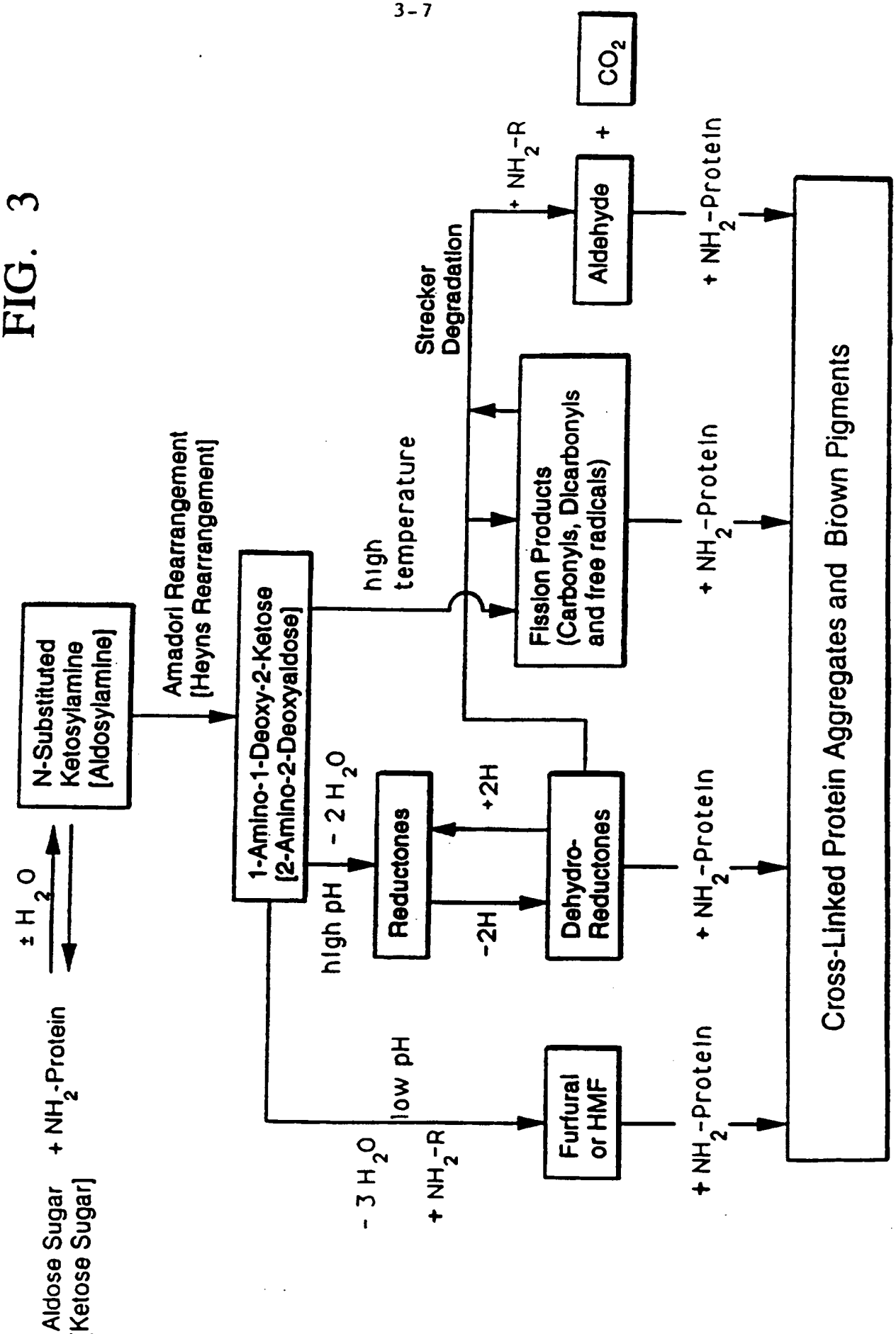

Aldose Sugar [Ketose Sugar]
± H2O
+ NH2-Protein
N-Substituted Ketosylamine [Aldosylamine]
Amadori Rearrangement [Heyns Rearrangement]
1-Amino-1-Deoxy-2-Ketose [2-Amino-2-Deoxyaldose]
high pH
- 2 H2O
Reductones
+2H
-2H
Dehydro-Reductones
low pH
- 3 H2O
+ NH2-R
Furfural or HMF
high temperature
Fission Products (Carbonyls, Dicarbonyls and free radicals)
Strecker Degradation
+ NH2-R
Aldehyde
+
CO2
+ NH2-Protein
+ NH2-Protein
+ NH2-Protein
+ NH2-Protein
Cross-Linked Protein Aggregates and Brown Pigments

FIG. 4

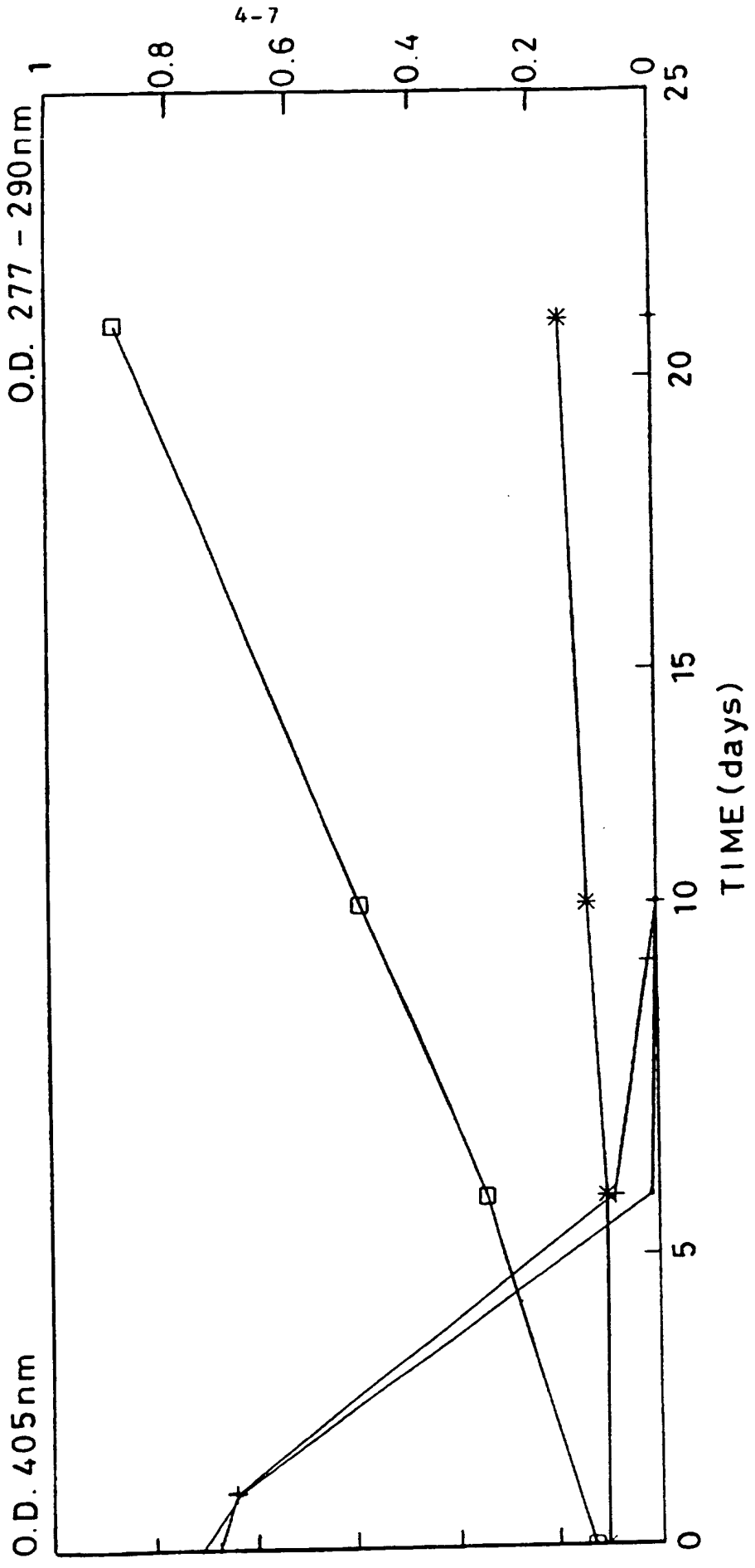


FIG. 5A

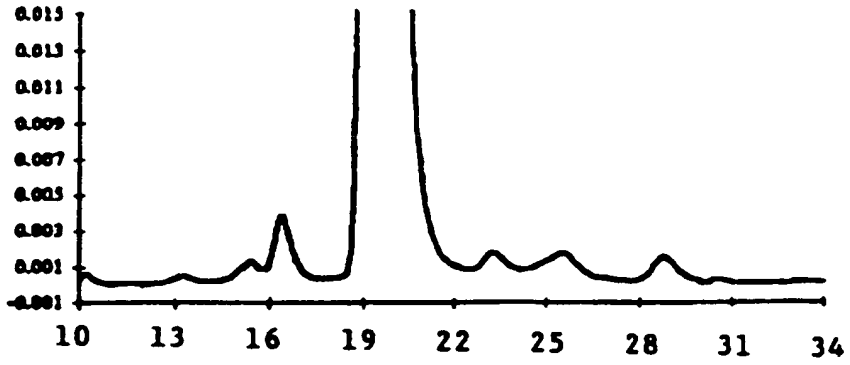


FIG. 5B

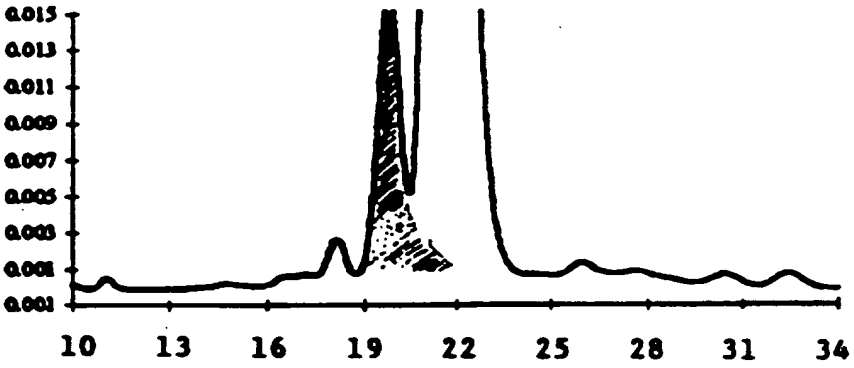


FIG. 5C

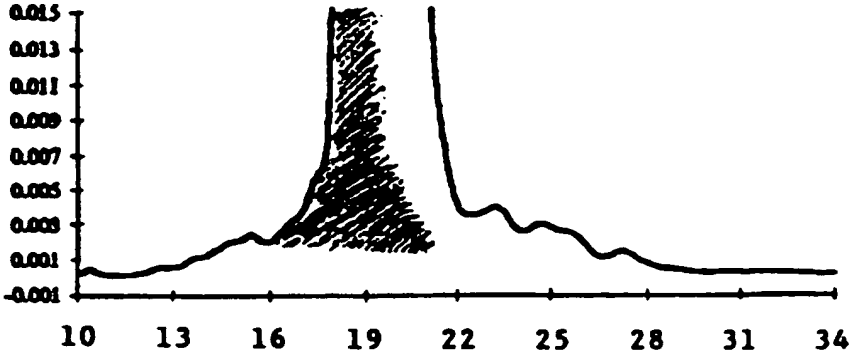


FIG. 5D

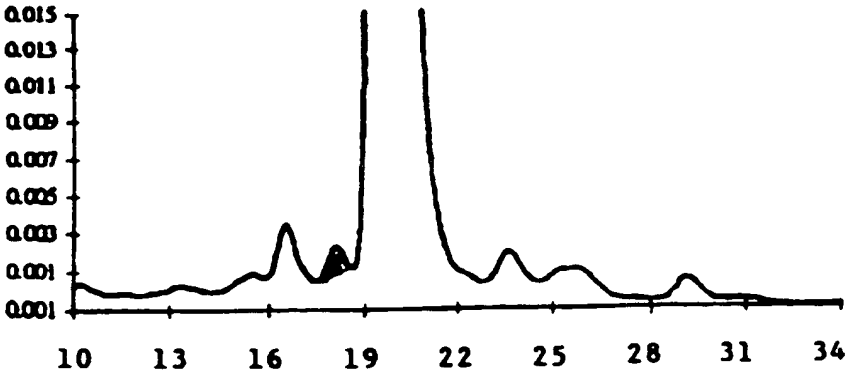


FIG. 6a

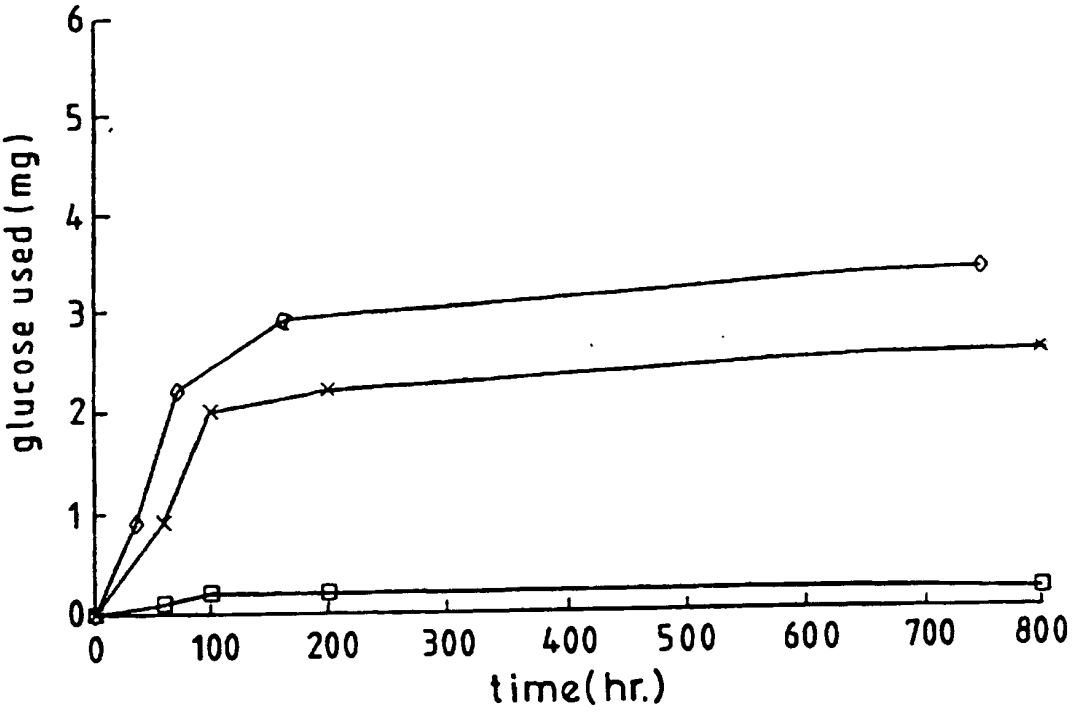

glucose used (mg)
0
1
2
3
4
5
6
0
100
200
300
400
500
600
700
800
time(hr.)

FIG. 6b

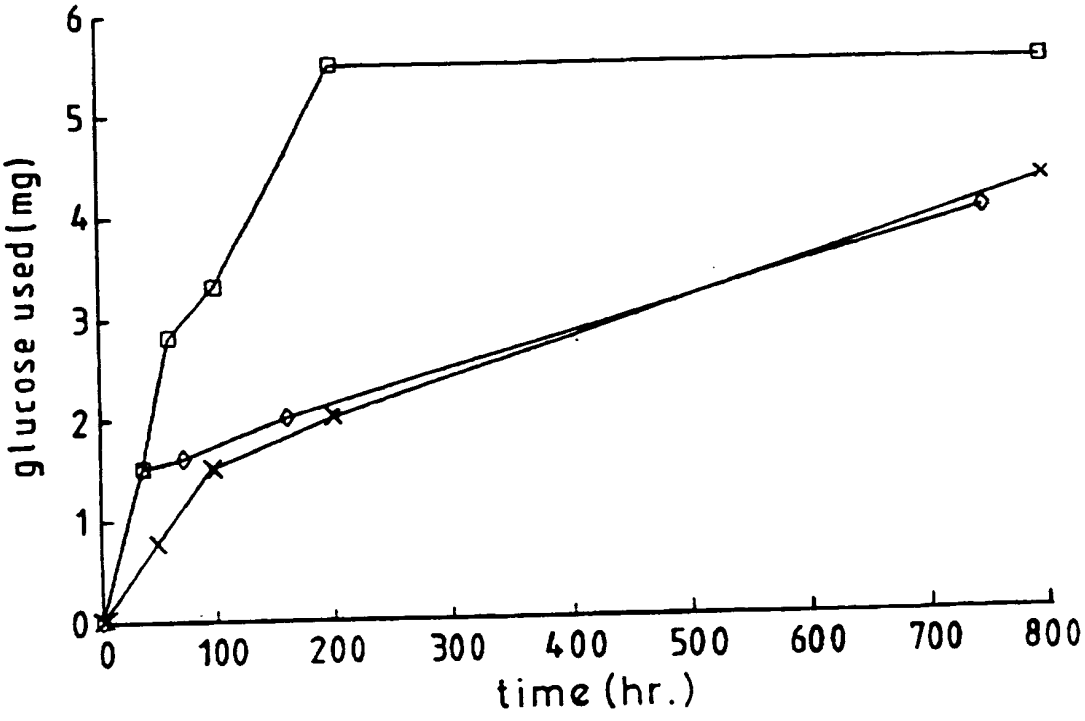
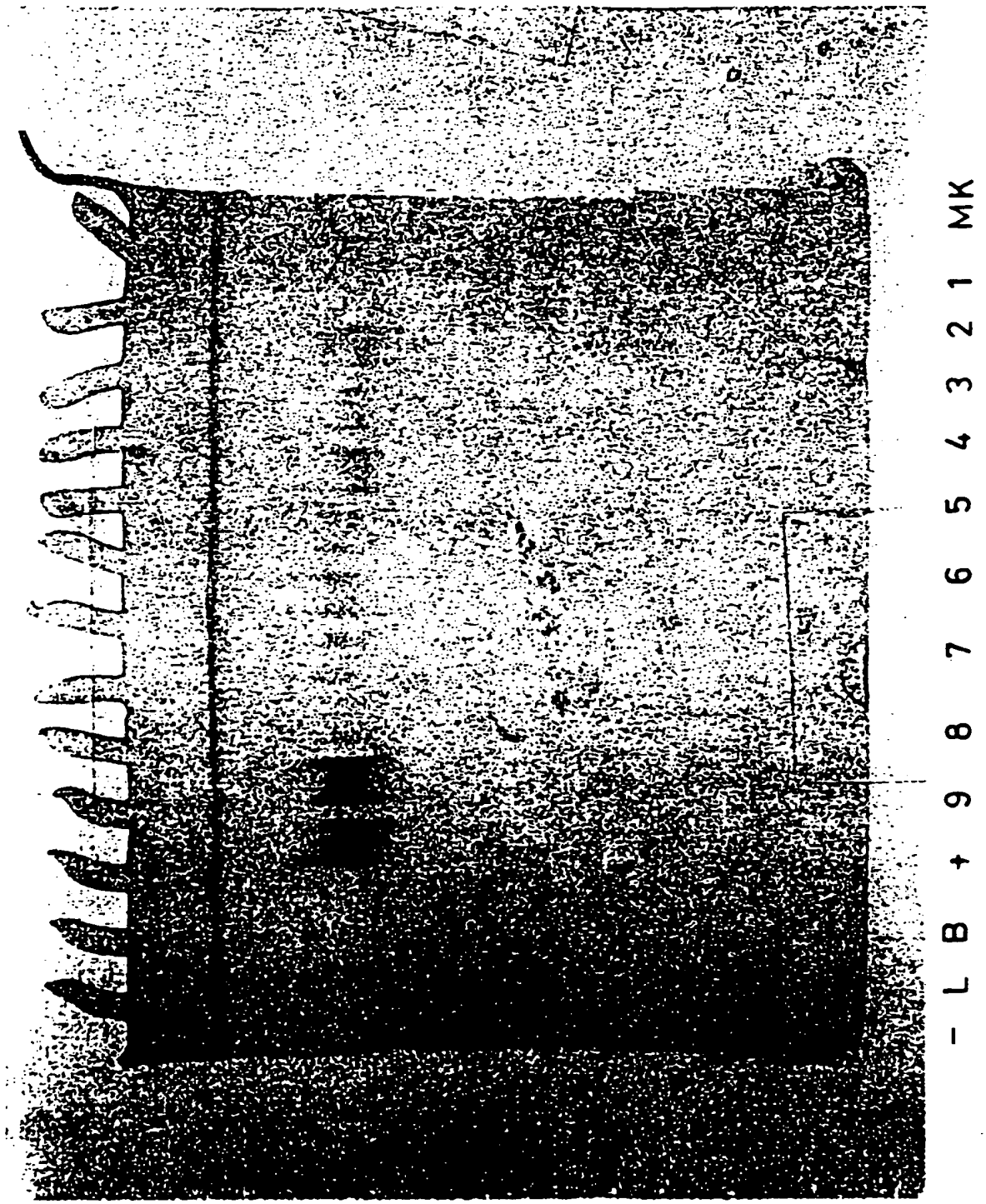

glucose used (mg)
0
1
2
3
4
5
6
0
100
200
300
400
500
600
700
800
time (hr.)

FIG. 7



A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/14 A61K9/19 A61K47/26 A61K47/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 600 730 (KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU KAGAKU KENKYUJO) 8 June 1994 see page 5, line 54 - page 6, line 9 see page 16, line 32 - line 44	1-95
A	EP,A,0 090 356 (TAKEDA CHEMICAL INDUSTRIES, LTD.) 5 October 1983 see page 1, line 1 - page 3, line 13	1-95
A	US,A,4 371 557 (OPPY ET AL.) 1 February 1983 see the whole document	1-95
A	EP,A,0 222 313 (THE ROCKEFRLLER UNIVERSITY) 20 May 1987 see page 2, line 2 - page 6, line 10	1-95

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 December 1995	20.12.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Benz, K

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-600730	08-06-94	JP-A-	6170221	21-06-94
		AU-B-	5203393	16-06-94
		CA-A-	2110331	03-06-94
		CN-A-	1094054	26-10-94
EP-A-90356	05-10-83	JP-C-	1583710	22-10-90
		JP-B-	2002916	19-01-90
		JP-A-	58164683	29-09-83
		US-A-	4547377	15-10-85
US-A-4371557	01-02-83	CA-A-	1160891	24-01-84
		JP-C-	1661445	19-05-92
		JP-B-	3023136	28-03-91
		JP-A-	57144946	07-09-82
EP-A-222313	20-05-87	US-A-	4758583	19-07-88
		AT-T-	112165	15-10-94
		AU-B-	610056	16-05-91
		AU-B-	6495886	21-05-87
		CA-A-	1294218	14-01-92
		DE-D-	3650080	03-11-94
		DE-T-	3650080	16-02-95
		ES-T-	2061435	16-12-94
		JP-A-	3103163	30-04-91
		JP-B-	6067827	31-08-94
		JP-A-	62142114	25-06-87
		US-A-	5316754	31-05-94
		US-A-	4908446	13-03-90
		US-A-	5468777	21-11-95
		US-A-	5175192	29-12-92
		US-A-	5140048	18-08-92
		US-A-	5106877	21-04-92
		US-A-	5114943	19-05-92
		US-A-	5258381	02-11-93
		US-A-	5218001	08-06-93
		US-A-	5202424	13-04-93
		US-A-	5238963	24-08-93
		US-A-	5254593	19-10-93
		US-A-	5221683	22-06-93
		US-A-	5334617	02-08-94

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-222313		US-A- 5356895	18-10-94
		US-A- 5243071	07-09-93
		US-A- 5262152	16-11-93
		US-A- 5272176	21-12-93
		US-A- 5399560	21-03-95
		US-A- 5318982	07-06-94
		US-A- 5358960	25-10-94
		US-A- 5326779	05-07-94
		US-A- 5272165	21-12-93

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)